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Analysis of Multigenerational Behavioral Effects of Dietary Benzo[a]pyrene Exposure in Adult Zebrafish

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ANALYSIS OF MULTIGENERATIONAL BEHAVIORAL EFFECTS OF DIETARY BENZO[A]PYRENE EXPOSURE IN ADULT ZEBRAFISH

By
Mary Beth Gillespie

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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ABSTRACT
MARY BETH GILLESPIE: Analysis of Multigenerational Behavioral Effects of Dietary Benzo[a]pyrene Exposure in Adult Zebrafish
(Under the direction of Dr. Kristine Willett)

Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon that is linked to negative reproductive and developmental effects in humans and animals. Because BaP is carcinogenic, and its continued presence in the environment allows it to be inhaled and ingested, better understanding of the effects of BaP is needed. To determine the behavioral effects of BaP exposure, zebrafish were used as a model. Wild-type zebrafish (5D) underwent two separate 21-day dietary exposures to 2.5 and 25 μg BaP/g fish to compare how BaP exposure affects locomotor activity. Following the dietary exposure, fish were mated to obtain and raise the F1 generation to 4 or 7 mpf (months post fertilization) to determine multigenerational effects of BaP on behavior. BaP is a ligand for the aryl hydrocarbon receptor (AHR in humans; Ahr in fish), which, in turn, mediates some of BaP's adverse outcomes (e.g., metabolic activation of a DNA reactive intermediate). Previous research has suggested that not all of BaP-mediated developmental defects are Ahr-dependent. To isolate the Ahr-dependent adverse outcomes, $Ahr2^{OSU1}$ zebrafish, which lack Ahr2, were exposed to 25 μg BaP/g fish to compare responses in Ahr null versus wild-type animals. Behavior in the open field test was analyzed to measure locomotor activity and assess anxiety-like behavior. In the F0 5D strain, no significant behavioral effects of dietary BaP exposure were observed. Adult F1 female offspring of parents exposed to 25 μg BaP/g fish had a significant increase in both distance traveled and time spent mobile when compared to controls. F1 behavioral effects were not significantly different in

males or when only one parental sex was exposed. Open field behaviors were not significantly different between control 4 mpf 5D and 4 mpf Ahr-null zebrafish. However, in the F0 Ahr2^{OSU1} strain, total distanced traveled was significantly decreased in males, but not females, following BaP exposure. The F0 Ahr2^{OSU1} fish did not reproduce, so F1 assessments could not be done. Overall, our results suggest that BaP behavioral impacts are sex-dependent and persistent in F1 adults, and behavioral changes in controls, as well as behavioral changes due to BaP, are not Ahr-dependent.

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LIST OF ABBREVIATIONS

AHR	Aryl Hydrocarbon Receptor
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
BaP	Benzo[a]pyrene
CYP	Cytochrome
DOHaD	Developmental Origins of Health and Disease
hpf	Hours post fertilization
LCD	Liquid-Crystal Display
mpf	Months post fertilization
PAH	Polycyclic Aromatic Hydrocarbon
ppm	Parts per Million
TCDD	2, 3, 7, 8-Tetrachlorodibenzodioxin

1. INTRODUCTION

1.1 Benzo[a]pyrene

Benzo[a]pyrene (BaP, **Figure 1**) is a polycyclic aromatic hydrocarbon (PAH) that is present in the environment as a result of the incomplete breakdown of organic compounds, notably petroleum-based fuels and coal (Latimer & Zheng, 2003). Cigarette smoke, grilled and broiled foods, as well as industrial processes are common sources of BaP (Services, 1999). Due to this, inhalation and ingestion are the main routes by which humans are exposed. BaP is categorized as a Group 1 carcinogen in humans, which is indicative of the highest cancer-causing potential (*Agents Classified by the IARC Monographs, Volumes 1–128*, 2021). Additionally, BaP exposure negatively affects reproduction and development in humans and animals (Jeng et al., 2015; Patel et al., 2016; Perera et al., 2006). In particular, maternal exposure to PAHs has been tied to low birth weight in humans (Siddiqui et al., 2008). Children have also been found to have a moderate delay in cognitive development at the age of 3 due to maternal PAH exposure (Perera et al., 2006). In laboratory experiments using fish, developmental BaP exposure adversely affected larval behavior and impaired adult learning and memory (Knecht et al., 2017). BaP exposure significantly decreased locomotor activity in zebrafish, including velocity and total distance traveled (Das et al., 2020). In contrast, BaP exposure induced hyperactivity in rats and impaired motor and cognitive behavior (Hawkey et al., 2019; Maciel et al., 2014; Patel et al., 2016). Despite studies indicating the negative effects

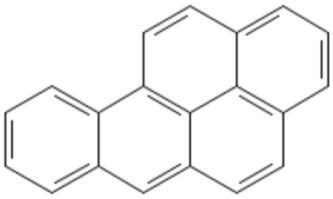

Figure 1: Structure of Benzo[a]pyrene

of BaP exposure, little is known about the molecular mechanism underlying these adverse behavioral outcomes. Because of the ubiquity of BaP and other PAHs in the environment and the negative effects of exposure to PAHs, further research into the possible multi- and transgenerational effects and the mechanistic pathway of BaP are needed.

1.2 Molecular Mechanisms of BaP

In order for BaP to induce negative effects, BaP must be bioactivated to reactive metabolites. One of the molecular pathways by which BaP metabolism is facilitated is by activation of the aryl hydrocarbon receptor (AHR in humans; Ahr in fish) pathway (Genies et al., 2013; Souza et al., 2016). The AHR is a ligand-activated transcription factor that is highly conserved across various animal phyla (Hahn et al., 2017). Before ligand binding, AHR is located in the cytosol, but upon binding with a ligand, such as BaP, the AHR is translocated to the nucleus. There, it will heterodimerize with the aryl hydrocarbon receptor nuclear translocator (ARNT) and form the ligand-AHR-ARNT complex. This complex works to regulate gene transcription, and then the complex will disassociate, and the AHR will return to the cytosol. The AHR-associated molecular pathways serve important roles at the cellular and molecular levels, including signaling pathways important for cell proliferation, the cell cycle, cell morphology, cell adhesion, and cell migration (Mulero-Navarro & Fernandez-Salguero, 2016). Pollutants like BaP and 2, 3, 7, 8-tetrachlorodibenzodioxin (TCDD) that bind to and activate AHR lead to adverse effects in wildlife and humans. Activation of AHR results in the induction (upregulation) of cytochromes P450 (CYPs) which, in turn, are responsible for metabolically activating BaP into toxic metabolites (Genies et al., 2013; Souza et al., 2016). One of the aims of our study was to test whether AHR plays a role in BaP's behavioral effects.

Inappropriate activation of the AHR can result in disadvantageous developmental and cognitive effects (Schneider et al., 2014). One of the approaches to understand the physiological roles of biological receptors is to genetically engineer animals without the receptor and compare these knockout animals with wild-type animals. AHR-null mice, although capable of becoming pregnant, have impaired fertility due to reduced fecundity as well as decreased survival during the period of pregnancy and lactation (Abbott et al., 1999). Similarly, in zebrafish, Ahr-null fish have reduced fecundity, abnormal follicular and oocyte development, neuromuscular and/or sensory growth, and growth and survival of offspring (Garcia et al., 2018). Ahr2-null adult zebrafish also have abnormal skeletal bone structures, damaged fins, and impaired behavioral responses, suggesting that Ahr2 affects neuromuscular and/or sensory system development (Garcia et al., 2018). Larval Ahr2-null zebrafish have abnormal behavioral responses to light, and adult Ahr2-null zebrafish have altered startle response and predator avoidance (Garcia et al., 2018).

1.3 Zebrafish Model

Zebrafish represent a model organism to study BaP toxicity mechanisms of action and their use provides a number of scientific advantages, including their well-conserved genomes, cell types, tissues, and organ systems with other animals (Garcia et al., 2016; Howe et al., 2013). Their rapid life cycle, high fecundity, transparent development, and ability to be genetically altered also contribute to zebrafish being useful models (Tierney, 2011). In zebrafish, there are three forms of the Ahr (Ahr1a, Ahr1b, and Ahr2), but Ahr2 is the primary ortholog for mammalian AHR (Hahn et al., 2017). Zebrafish also serve as a good model for behavior due to their activity being a “viable endpoint for detecting neurological impairments received during development” (Tierney, 2011). Zebrafish are easily handled due to their larvae being small and

husbandry costs relatively low. Their large number of offspring and rapid larval development allow for neurotoxic responses to be assessed at an early developmental stage (Kimmel et al., 1995). Depending on the chemical concentration and the developmental exposure time, behavioral screenings can be done to show chemical-mediated increases and/or decreases in locomotor activity. Significant alteration in activity response due to exposure to the chemical can indicate neurological changes in the fish (Tierney, 2011). Spontaneous swimming tests or open field tests allow for chemical exposures to be compared to control conditions in order to determine effects in locomotion (Fitzgerald et al., 2021). Thigmotaxis, the tendency for zebrafish to swim in the periphery of the well, can be a measure of anxiety behavior and is detectable through spontaneous swimming tests (Norton, 1995).

1.4 Study Goals

Due to cognitive impairments noted in humans following parental exposure to PAHs, we wanted to determine if a dietary or a preconceptional BaP exposure would have negative and persistent adverse effects on behavior.

The two main goals of this study were to:

1. Evaluate how a preconceptional BaP exposure results in changes to the behavioral responses in adult zebrafish in the F0 (dietarily-exposed) and F1 (parental exposure only) generations.
2. Assess the role of AHR in mediating changes in behavioral responses following BaP dietary exposure.

Our hypotheses were:

1. BaP-induced behavioral impacts will be sex-dependent.
2. Behavioral impacts of preconceptional BaP exposure will be persistent in F1 adults.
3. Behavioral changes in controls are not AHR-dependent.
4. Behavioral changes due to BaP are not AHR-dependent.

2. MATERIALS AND METHODS

2.1 Zebrafish Husbandry

The wild-type (5D) and Ahr2^{OSU1} zebrafish were acquired from Dr. Robyn Tanguay at Oregon State University, and all of the fish were raised following the approved IACUC protocol. See **Figure 2** for representative pictures of the wild-type and Ahr2-null zebrafish. The fish were kept in Aquatic Habitats ZF0601 Zebrafish Stand-Alone System with zebrafish water (pH 7.0-7.5, 60 parts per million (ppm) Instant Ocean, Cincinnati, OH) at 25-28 °C. Twice daily, fish were fed Gemma 300 micro food (Skretting USA, UT). Fish were selected as breeders if they were found to be sexually mature and lacking any deformities or signs of disease.

The fish selected for breeding, in a 1:1 ratio of males to females, were placed in breeding tanks the evening before egg collection. When the lights turn on, the fish lay their eggs, and an hour later, the eggs were collected. A sieve was used to collect the eggs that fell to the bottom of the breeding tank through the protective gate by pouring the water through the sieve. The eggs were then cleaned and transferred to a petri dish. There they were raised in embryo water (pH 7.5, 60 ppm Instant Ocean, 14:10 light dark cycle) in an incubator at 28 °C. Every day, a transfer pipette was used to remove dead and/or unfertilized eggs as well as debris. The larvae were raised for 4-7 months post fertilization (mpf) and served as the F0 generation for the exposures.

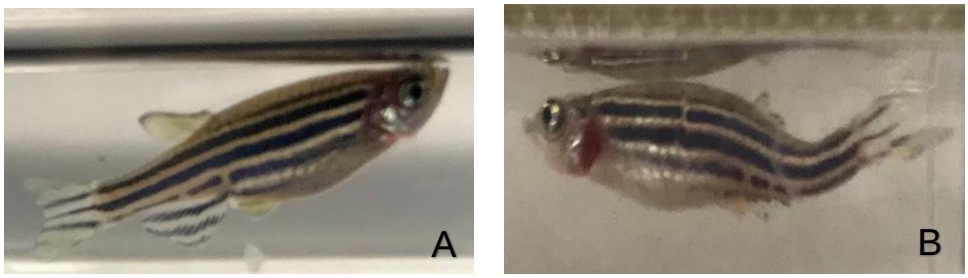

Figure 2: Representative Pictures of 5D Wild-type (A) and Ahr2^{OSU1} (B) Adult Zebrafish. Ahr2 null animals typically display the fin deformities and curvature of the body axis.

2.1.1 Genotyping of Ahr2^{OSU1}

To confirm the knockout of Ahr2, adult Ahr2^{OSU1} fish were genotyped. Buffered MS-222 (150 mg/L) was used to anesthetize the fish and a small portion of the fin was clipped. Following lysis and protein degradation, DNA was extracted from the fin. The PCR primers used were Forward 5'-TTC AAC AGT CCT CCT TAA GAA CG-3' and Reverse 5'- TGT AAA ATA ACA ACA TAA CTT GGC CC-3' (Garcia et al., 2018). The PCR product was then restriction enzyme digested with Nde1 (New England Biolab) and run on a gel to determine if each fish was homozygous recessive.

2.2 Exposures

2.2.1 Parental Dietary Exposure

Either acetone-treated or BaP- treated (2.5 or 25 µg BaP/g fish, equivalent to 125 or 1250 µg BaP/g food, respectively) Tetramin flake food was fed to sexually mature (4-7 mpf) zebrafish (5D and Ahr2^{OSU1}). Acetone was purchased from Fisher Scientific (Fair Lawn, NJ) and BaP from Supelco Analytical (Belfonte, PA). To prepare the treated flake food, 24 g of flake food was spiked with 18 ml of acetone containing BaP (0, 0.1667, or 1.667 µg BaP/µL). This was equivalent to

nominal BaP concentrations in the food of 0, 125.1, or 1251 µg BaP/g food. Immediately, the spiked flakes were rotovapped to dryness, and the flakes were then stored at room temperature in amber vials. Paired (2 male x 2 female) zebrafish in five replicate tanks per treatment group (N=5 replicate tanks for a total 20 fish/group) were allowed to acclimate for a week while maintained at 25.5-28°C. During this time, the fish were fed twice daily with TetraMin® Tropical Flakes and Gemma 300 micro food. During the exposure, fish were fed 1% body weight twice daily of the corresponding dose of control- or BaP-treated flake food and once daily Gemma 300 micro food for 21 days. At the end of day 21, a cross-over breeding design was implemented to assess sex-specific contributions into the following treatment groups: Control M x Control F, Control M x BaP F, BaP M x Control F, and BaP M x BaP F. Eggs were collected to determine reproductive success on days 22 and 23 (while fed only BaP-free food). On day 24 (females) and day 25 (males), behavior in an open field test was performed as described in section 2.3 below.

2.2.2 Extractions and Chemical Analysis

The extraction and chemical analysis of the flake food has been previously described in (Corrales et al., 2014). Actual BaP concentrations of the treated flakes were: 78.3 ± 1.4 µg BaP/g flake and 708 ± 26 µg BaP/g flake for the 5D exposure and 840 ± 31 µg BaP/g flake for the $Ahr2^{OSU1}$ exposure. Percent recoveries ranged from 125-240%.

2.3 Behavior Analysis

At the end of the exposure, 10 fish/sex/group were acclimated for 20 min to a darkened behavioral testing room (27-28°C) prior to open field behavioral assessment. Individual fish were transferred to a water-filled bucket (diameter of 23 cm and a depth of 25 cm; Figure 3a). The fish were allowed to swim freely and explore for 5 minutes. Meanwhile, their response was

captured on overhead video by Noldus Ethovision 14 software. The testing area was lit to 9 Lux. When the trial was complete, the fish were removed from the open field arena and placed in a holding container until euthanized. For analysis, the swim arena was divided into two regions- periphery (outer 50% of the arena) and center (inner 50% of the arena (11.5 cm)) (Figure 3b). Distance, mobility, and time spent in each region of the arena was then calculated by Ethovision and decoded by a blinded observer. Distance was calculated by the Ethovision software as the distance traveled by the center of the subject for the five-minute duration. Mobility was defined as the percentage changed pixels of the detected subject between current time frame and the previous time frame The video tracks were manually cleaned in order to eliminate gaps in distance and speed in order to reduce inconsistencies in the software's tracing due to flashing, shadows, or water movements.

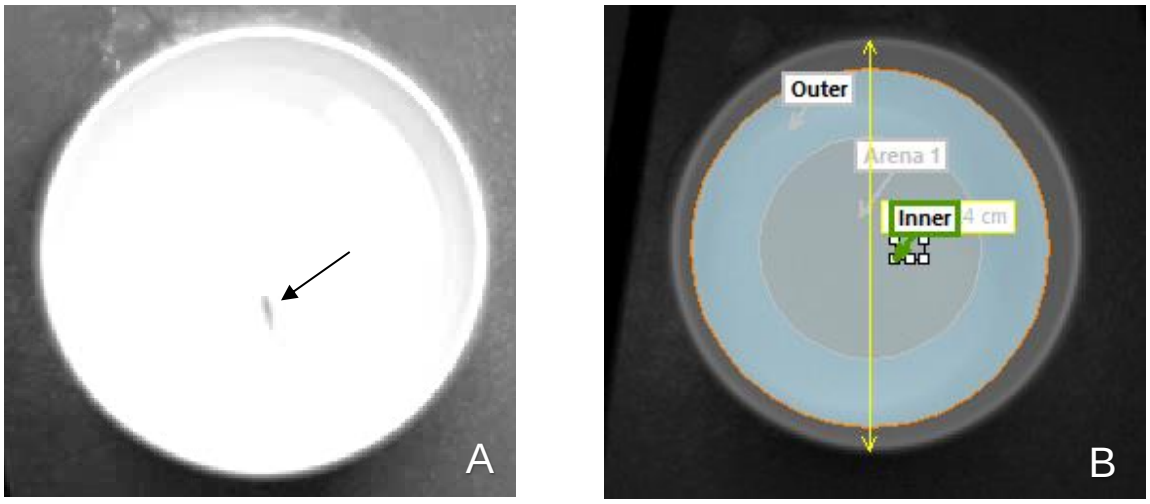

Figure 3: Fish in the Open Field Test Bucket (A) and Open Field Test Arenas (B). Image (A) demonstrates the bucket in which the fish were placed for analyzing behavior with the arrow indicating the fish, and image (B) demonstrates the different zones of the bucket used to determine time in the periphery (outer) during behavioral analysis.

2.4 F1 Generation

On days 22 and 23 of F0 exposure, fish were mated from each treatment group. This was to obtain and subsequently raise the F1 generation. At 120 hpf, larvae were randomized per treatment group and transferred to 3L tanks and raised until 4-7 mpf. F1 endpoints were collected as with the F0 generation.

2.5 Statistics

We analyzed data using Sigma Plot 14.0 software and presented as box and whisker plots. For adult behavior, males and females were analyzed separately. When only two treatment groups were compared, as in the F0 fish, an unpaired t-test was utilized to determine statistical significance. For the F1 fish, one-way ANOVA followed by Dunnett's post hoc test was used to determine statistical significance between treatment groups and control. Statistical significance was determined if $p<0.05$.

3. RESULTS

Three different exposures to BaP were studied. The 2.5 μg BaP/g fish exposure at 6-7 mpf was conducted to determine how this concentration of BaP affected the total distance (cm), time spent mobile (%), and total time in the periphery (%) for the 5D zebrafish. Total time in periphery was analyzed because the time spent in the periphery can be indicative of anxiety-like behavior. Time spent in the periphery was indicated by heatmaps (examples in Figure 4) of the wild-type and Ahr-null zebrafish.

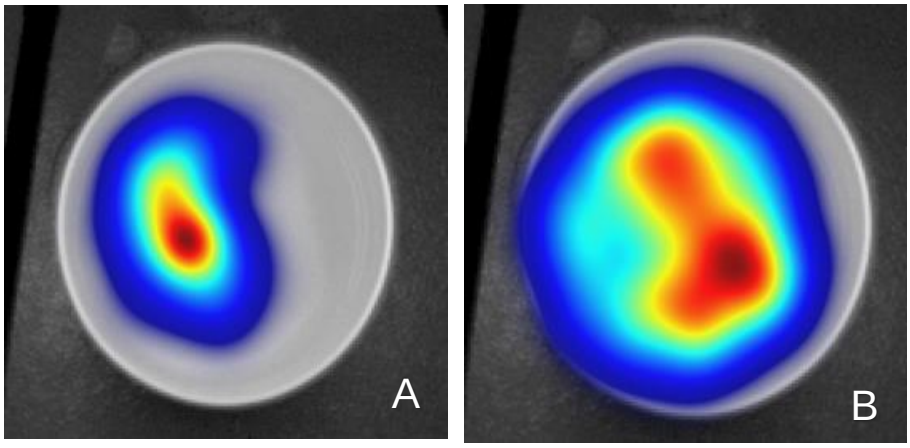

Figure 4: Control (A) and BaP Exposed (B) Ahr- null Zebrafish Heatmaps. The heatmaps above indicate the region of the bucket in which the fish spends the most time, with red indicating the most time and blue indicating the least time. These heatmaps were used to analyze the time each fish spent in the periphery. Total assay time was five minutes.

Due to the lack of F1 larval toxicities observed following the 2.5 μg BaP/g fish exposure, a second higher dose exposure was conducted with 25 μg BaP/g fish at 4 mpf in the 5D zebrafish. To analyze the potential persistent multi-generational effects of these exposures, F1 crosses of the 2.5 and 25 μg BaP/g fish exposed fish were conducted. In order to explore the role of AHR in BaP-mediated changes in zebrafish behavior, a third exposure of 25 μg BaP/g fish was conducted at 4 mpf in Ahr2^{OSU1}zebrafish.

In the 2.5 μg BaP/g fish exposure, 5D zebrafish (6-7 mpf) were exposed to control (no BaP) or 2.5 μg BaP/g fish. Total distance (cm), time spent mobile (%), and total time in the periphery (%) are shown in Figure 5 for both control and exposed zebrafish. The low dietary BaP dose did not significantly alter any of the variables for the 5D F0 wild-type fish relative to controls, regardless of sex.

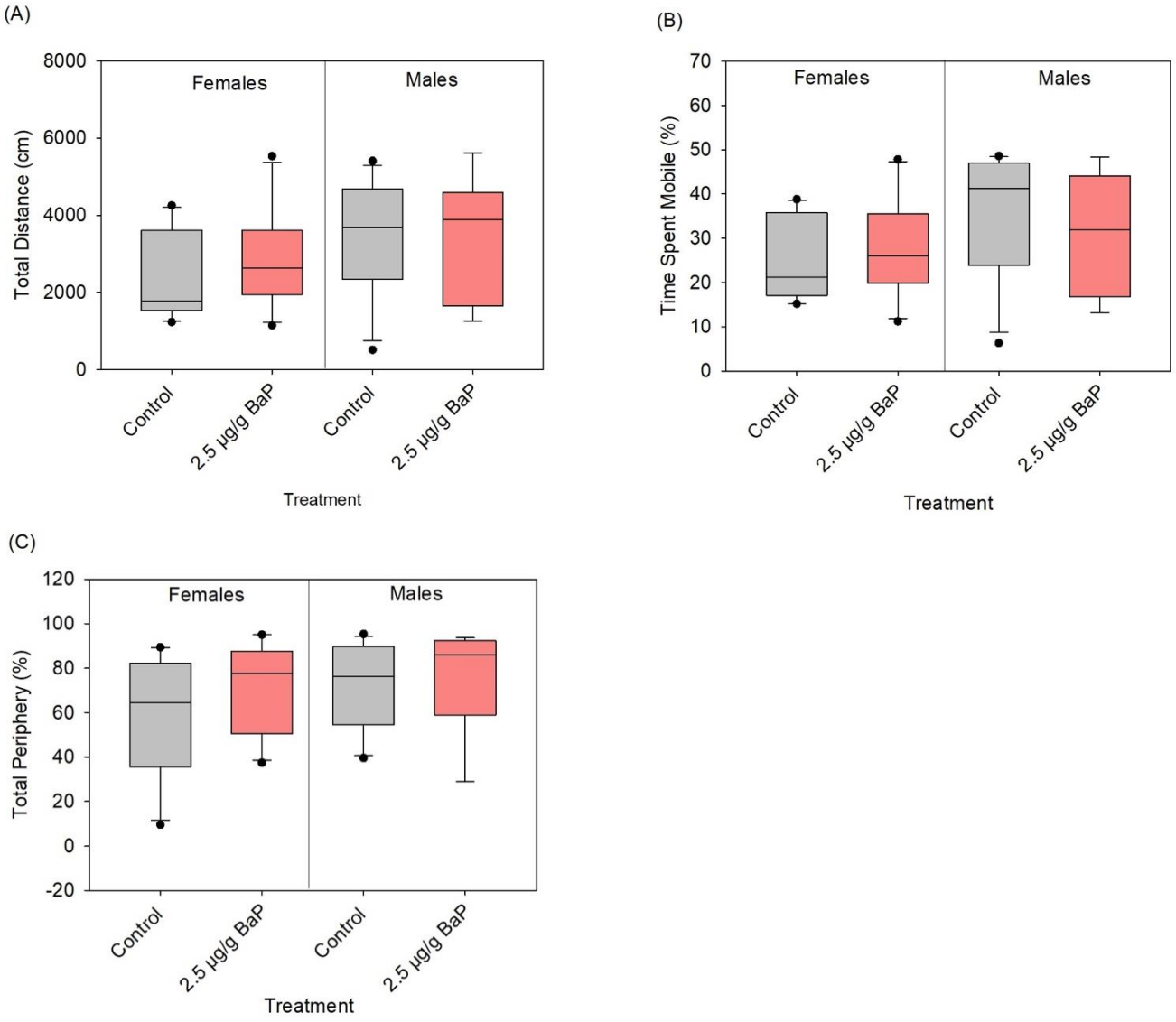

Figure 5: 5D F0 Adult Behavioral Effects Following 2.5 µg BaP /g Fish Dietary Exposure for 21 Days. Zebrafish adult behavior was analyzed using Noldus Ethovision 14.0 Software to record total distance (A), time spent mobile (B), and total time in the periphery (C) during the 5-minute period. Behavioral analysis was conducted at 7 mpf, following exposure to 2.5 µg BaP/g fish. Data were analyzed first to determine if behavior was significantly different between sexes with a t-test, and males (n=8-11) were not significantly different than females (n=10), but they were analyzed separately. Bars with the (*) symbol above them are significantly different ($p \leq 0.05$).

Due to no significant changes in the 2.5 μg BaP/g fish exposure relative to controls, a second exposure at a higher concentration was conducted. In the second exposure, 5D zebrafish (4 mpf) were exposed to control or 25 μg BaP/g fish in the diet. Total distance (cm), time spent mobile (%), and total time in the periphery (%) are shown in Figure 6 for both control and exposed zebrafish, and the results shown are the 5-minute segment of behavioral analysis. There was still no significant change in total distance, time spent mobile, and total periphery in the 25 μg BaP/g fish exposed zebrafish of either sex in comparison to the controls.

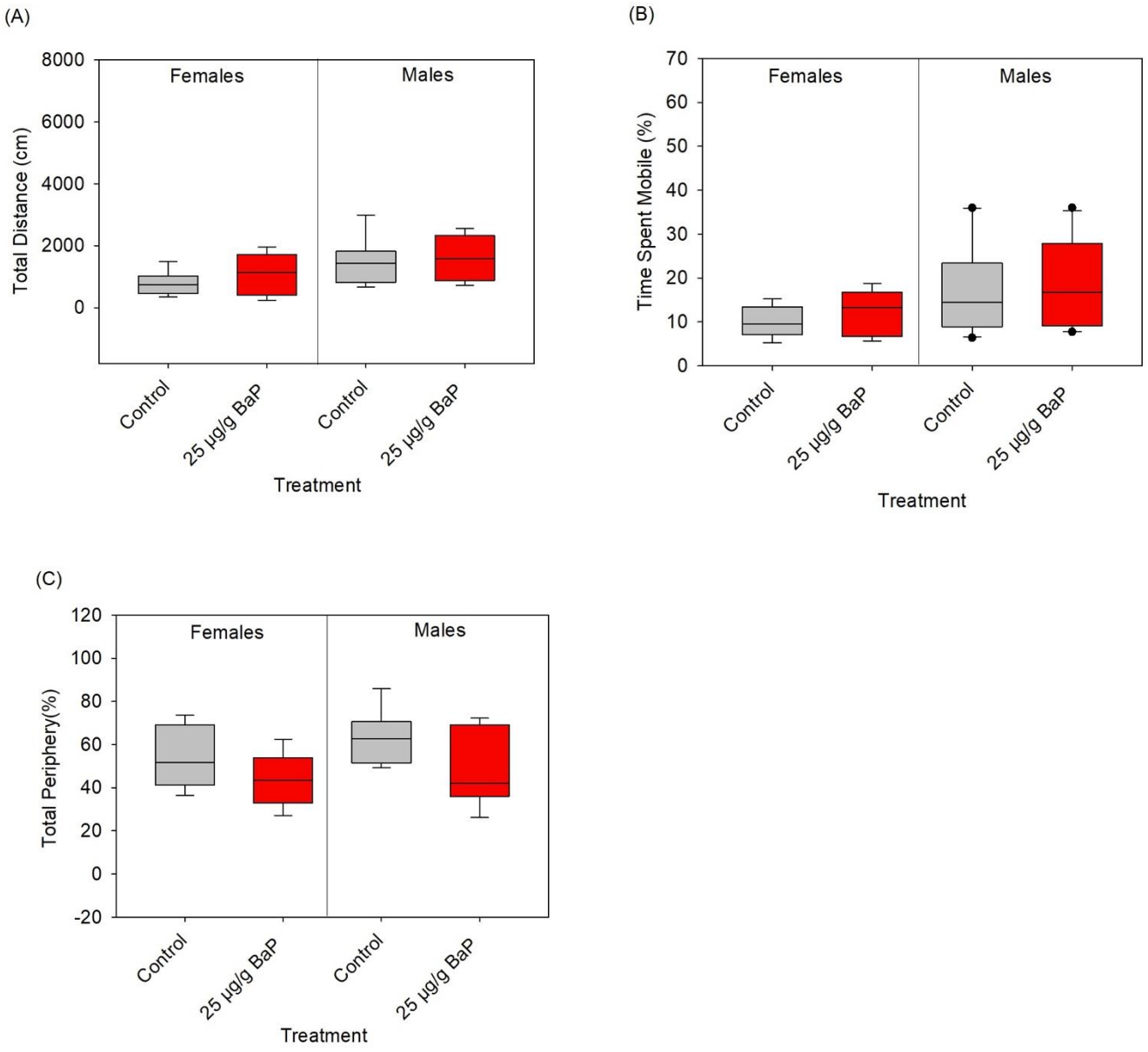

Figure 6: 5D F0 Adult Behavioral Effects Following 25 µg BaP /g Fish Dietary Exposure for 21 Days. Zebrafish adult behavior was analyzed using Noldus Ethovision 14.0 Software to record total distance (A), time spent mobile (B), and total time in the periphery (C) during the 5-minute period. Behavioral analysis was conducted at 4 mpf, following exposure to 25 µg BaP/g fish. Data were analyzed first to determine if behavior was significantly different between sexes with a t-test, and males (n=10) were significantly different than females (n=8) in regards to total distance traveled, and mobility. Therefore, the sexes were separated to determine if there were treatment effects with a t-test. Bars with the (*) symbol above them are significantly different ($p \leq 0.05$).

In order to analyze the multigenerational effects of BaP, on days 22 and 23 of F0 exposure, fish were mated from each treatment group using a cross-over design. This was to obtain and subsequently raise the F1 generation. F1 endpoints were collected similarly to the F0 generation, at 4 mpf (25 μg BaP/g fish exposed parents) or 7 mpf (2.5 μg BaP/g fish exposed parents). Total distance (cm), time spent mobile (%), and total time in the periphery (%) are shown in Figure 7 for each parental treatment cross: Control Male x Control Female, Control Male x BaP Female, BaP Male x Control Female, and BaP Male x BaP Female. As found in the F0 generation following exposure to 2.5 μg BaP/g fish, no significant effects were found in the F1 generation compared to the control group.

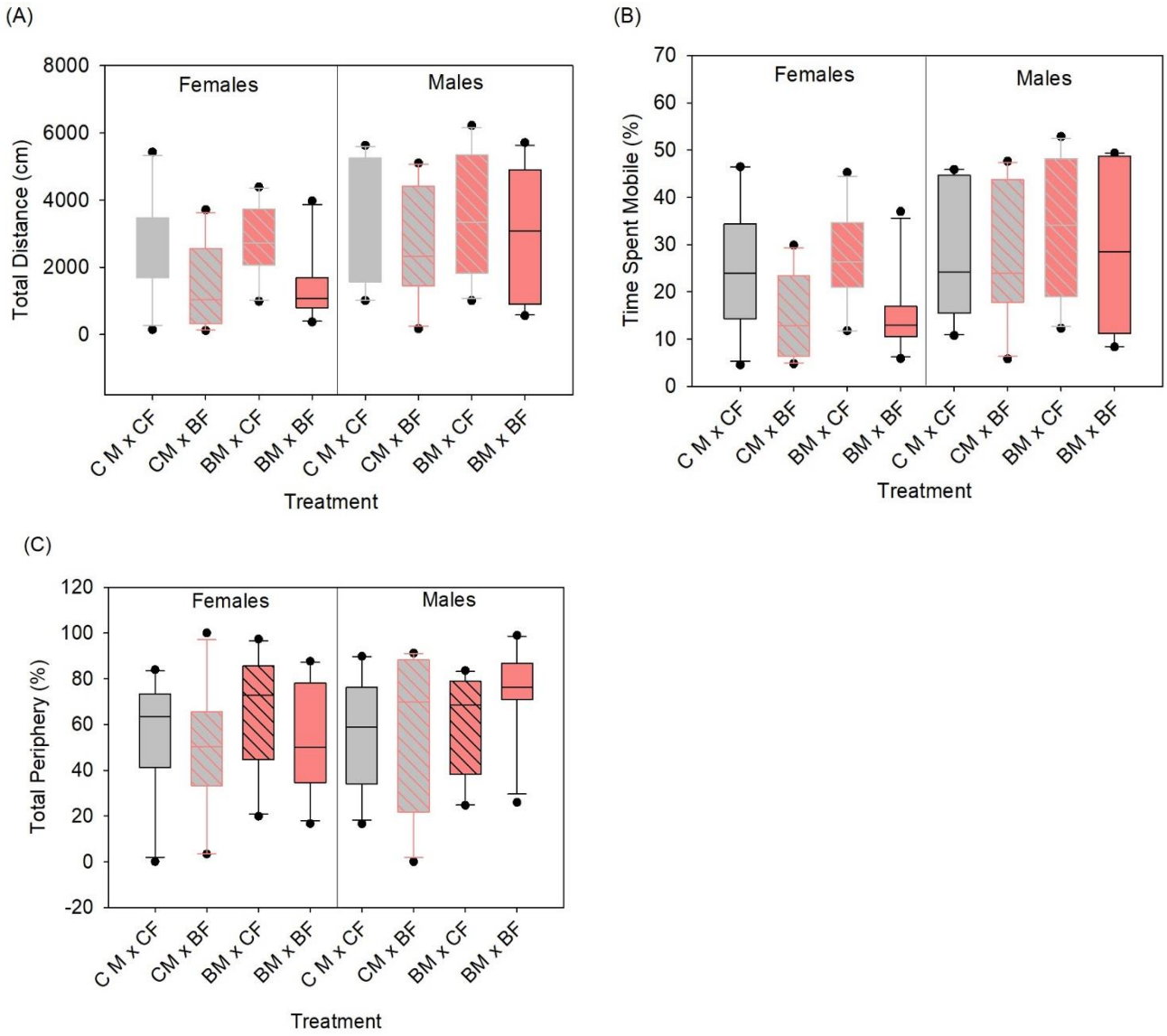

Figure 7: 5D F1 Adult (7 mpf) Behavioral Effects Following Parental 2.5 µg BaP/g Fish Dietary Exposure. Zebrafish adult behavior was analyzed using Noldus Ethovision 14.0 Software to record total distance (A), time spent mobile (B), and total time in the periphery (C) during the 5-minute period. Behavioral analysis was conducted at 7 mpf in the F1 generation, following parental exposure to 2.5 µg BaP/g fish. Data were analyzed first to determine if behavior was significantly different between sexes with a t-test, and males (n=10) were significantly different than females (n=10). Therefore, for these analyses the sexes were separated to determine if there were treatment effects. A one-way ANOVA test was run for further statistical analysis of significance followed by Dunnett's post hoc test if it was normally distributed. Bars with the (*) symbol above them are significantly different ($p \leq 0.05$). CM x CM refers to Control Male x Control Female. CM x BF refers to Control Male x BaP Female. BM x CF is BaP Male x Control Female, and BM x BF is BaP Male x BaP Female.

Following a parental exposure of 25 μg BaP /g fish, F1 female adults of the BaP M x BaP F group had a significant increase in the total distance traveled and the time spent mobile (Figure 8). Adult male F1 zebrafish behavior from any of the crosses in comparison to the control group was not significantly altered.

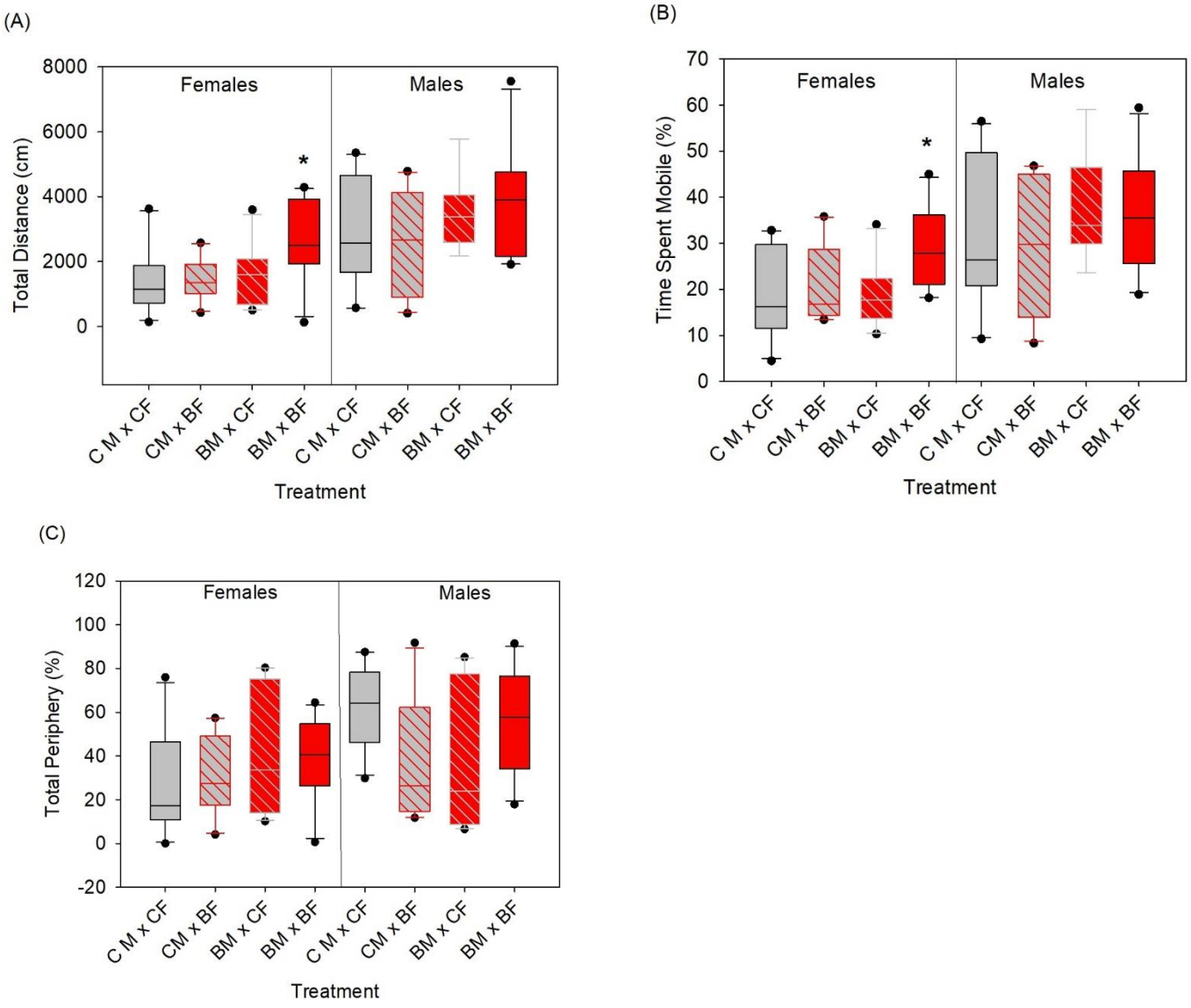

Figure 8: 5D F1 Adult (4 mpf) Behavioral Effects Following Parental 25 µg BaP/g Fish Dietary BaP Exposure. Zebrafish adult behavior was analyzed using Noldus Ethovision 14.0 Software to record total distance (A), time spent mobile (B), and total time in the periphery (C)) during the 5-minute period. Behavioral analysis was conducted at 4 mpf in the F1 generation, following parental exposure to 25 µg BaP/g fish. Data were analyzed first to determine if behavior was significantly different between sexes with a t-test, and males (n=9-10) were significantly different than females (n=10). Therefore, for these analyses the sexes were separated to determine if there were treatment effects. A one-way ANOVA test was run for further statistical analysis of significance followed by Dunnett's post hoc test if it was normally distributed. Bars with the (*) symbol above them are significantly different ($p \leq 0.05$). CM x CM refers to Control Male x Control Female. CM x BF refers to Control Male x BaP Female. BM x CF is BaP Male x Control Female, and BM x BF is BaP Male x BaP Female.

To study the impact of functional Ahr2 on behavior, $Ahr2^{OSU1}$ null zebrafish were used. The null animals are on the 5D background. Ahr-null controls and 5D controls (4 mpf) were compared and there was no significant difference between controls in any of the open field behaviors (total distance shown in Figure 9D). To test the hypothesis that BaP F0 dietary effects on behavior are AHR-mediated, 4 mpf $Ahr2^{OSU1}$ were exposed to control or 25 μg BaP/g fish diets for 17 days. Again, total distance (cm), time spent mobile (%), and total time in the periphery (%) during a 5-minute open field trial were analyzed, and the results are shown in Figure 9 A-C for both control and exposed zebrafish. Unlike with the wild-type 5D exposures, there was a significant difference when comparing the BaP to the control Ahr2 mutants. F0 Ahr2 null adult BaP-exposed males traveled significantly less total distance. However, there was not a significant change in either time spent mobile or the percent of time in the periphery for the exposed AhR null zebrafish of either sex in comparison to the control group. $Ahr2^{OSU1}$ exposed F0 did not reproduce; therefore, no F1 behavioral effects are reported here.

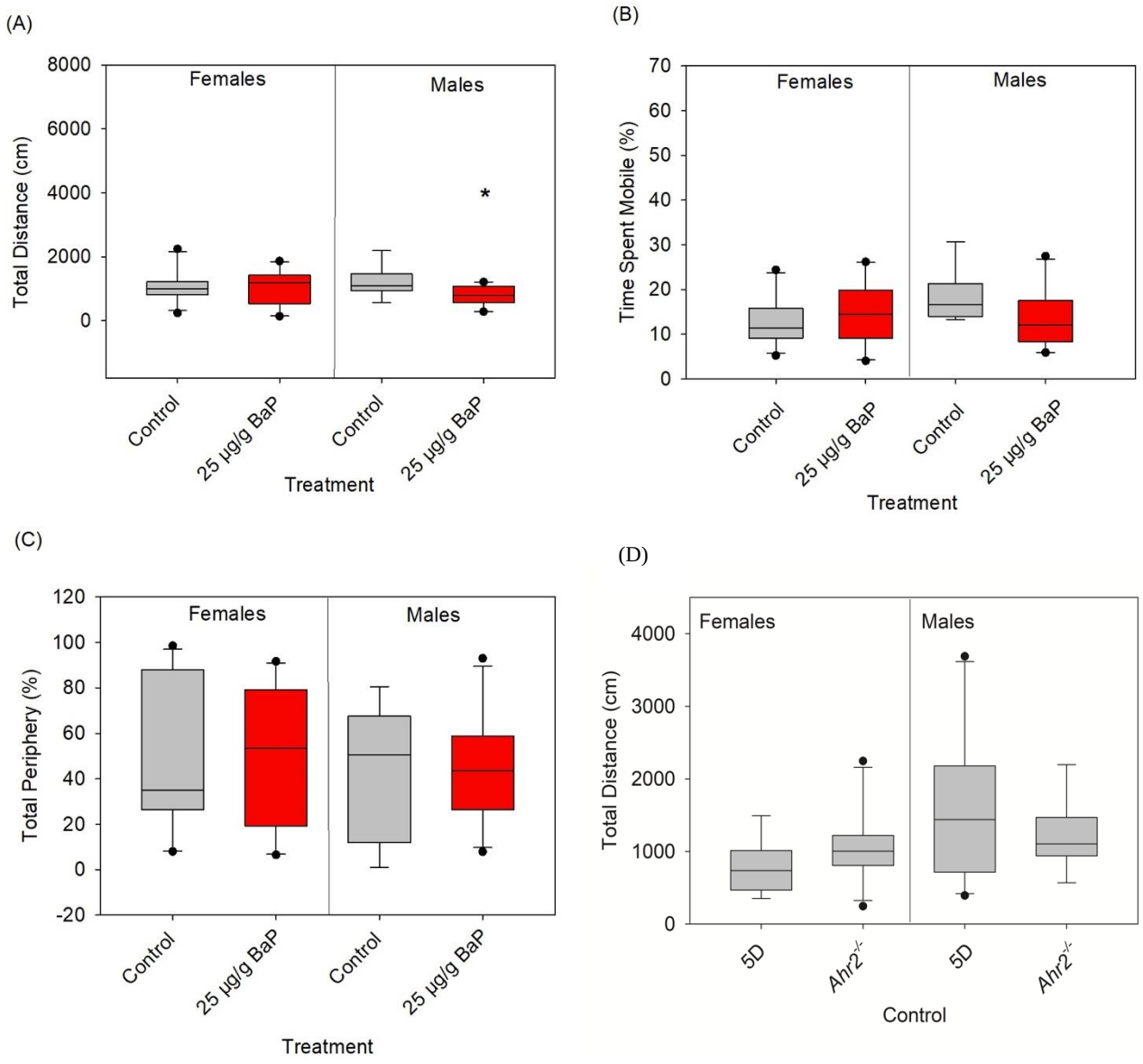

Figure 9: Ahr2^{OSU1} F0 Adult Behavioral Effects Following 25 µg BaP/g Fish Dietary Exposure for 17 Days. Zebrafish adult behavior was analyzed using Noldus Ethovision 14.0 Software to record total distance (A), time spent mobile (B), and total time in the periphery (C) during the 5-minute period. Image D) compares the total distance moved between 5D and Ahr-null control groups. Time spent mobile and total periphery control comparisons are not pictures. Behavioral analysis was conducted at 4 mpf, following exposure to 25 µg BaP/g fish. Data were analyzed first to determine if behavior was significantly different between sexes with a t-test, and males (n=9-10) were significantly different than females (n=10-11). Therefore, the sexes were separated to determine if there were treatment effects with a t-test. Bars with the (*) symbol above them are significantly different (p≤0.05).

4. DISCUSSION

The Developmental Origins of Health and Disease (DOHaD) hypothesis suggests that certain environmental exposures during critical developmental and growth periods can cause effects to an individual's short and long term health (Barker, 2007). For example, in a study done on 20 counties in Norway, Forsdahl used infant mortality as an index for measuring the standards of living and found a positive correlation in counties having high infant mortality during the cohorts' adolescent years and high mortality from arteriosclerotic heart disease when the cohorts were adults 40-69 years old. Forsdahl determined poverty during youth, resulting in poor living conditions, were positively correlated with the risk of dying from arteriosclerotic heart disease later in life (Forsdahl, 1977). In relation to PAH exposure, male coke-oven workers exposed to PAHs experienced decreased DNA integrity of sperm (Jeng et al., 2015), and maternal inhalation of PAHs was found to be associated with decreased cognitive development in offspring at the age of 3 (Perera et al., 2006). The discovery that pre-natal exposures can result in developmental effects in offspring resulted in our increased interest in better understanding the mechanisms by which preconceptional BaP exposure could cause toxicity.

Zebrafish were selected as a preconceptional exposure model in order to study the effects of BaP developmental exposure. In a previous study from our laboratory, it was established that BaP, through parental dietary exposure, resulted in multigenerational phenotypic larval deformities in F1, F2, and F3 generations. Mortality was also significantly increased in the F1 generation following parental dietary exposure to medium and high concentrations of BaP (Corrales et al., 2014). Following the establishment of multigenerational phenotypic effects of BaP, the two goals of this study were to (1) evaluate how preconceptional BaP exposure results in changes to the behavioral responses of F0 exposed adult zebrafish and their F1 offspring as

adults and (2) to assess the role of Ahr2 in mediating changes in adult behavioral responses due to BaP exposure. Our research will provide information on the molecular mechanisms for the development and potential multigenerational effects associated with exposure to BaP through diet.

Spontaneous swimming or open field tests are useful for detecting general defects in locomotion through the measurement of several parameters including swimming speed, time, and distance. Open field tests are also used to monitor thigmotactic behavior (Fitzgerald et al., 2021). In this study, zebrafish were placed in a bucket, which was used as the open field for analyzing behavior. There were different zones assigned to the regions of the bucket (center and periphery), and these zones were used to determine the time in the periphery. We analyzed the total distance traveled, total time spent mobile, and time spent in the periphery to determine if BaP induced anxiety-like behavior which is represented by thigmotaxis. These locomotor activity endpoints have become widely accepted in investigating the effects of different drugs and neurotoxins, including, but not limited to, amphetamines, cocaine, and nicotine (Irons et al., 2010; Levin et al., 2007; López-Patiño et al., 2008).

Previously, it was found that F1 AB line wild-type zebrafish parentally exposed to 0.21, 2.3, and 20 μg BaP/g fish had numerous developmental deformities as larvae (Corrales et al., 2014). For the purpose of our research on the role of Ahr2, 5D strain fish were selected for our exposures because the Ahr2 mutant fish are on the 5D background. Dose selection was based on our prior work in the AB line. The 5D F0 2.5 μg BaP/g fish exposure began at 6-7 mpf and behavior was recorded following 21 days of exposure. There was no significant change in total distance traveled, time spent mobile, and total time in the periphery for the exposed zebrafish in comparison to control. The malformations found in the larvae in the previous study were also not

present. This could be due to differences in the sensitivity of the AB and 5D strains which is not well documented due to poorly controlled background genetics in the zebrafish model and lack of characterization of strains (Crim & Lawrence, 2021). Due to the lack of findings from the low concentration exposure, a second exposure was conducted at a higher concentration of 25 μg BaP/g fish beginning on 5D zebrafish. However, similar to the lower concentration exposure, there were no significant changes in total distance traveled, time spent mobile, and total time in the periphery for the dosed zebrafish in comparison to the control in the F0 generation. The lack of significant changes in locomotor activity differs from what was previously found in a study by Das et al. (2020) following zebrafish BaP exposure, but the differences could be attributed to the differences in concentrations of BaP, route of exposure, as well as their assessment of locomotor activity by the novel tank diving test instead of the spontaneous swimming test.

To determine whether a preconceptional BaP exposure would cause lasting sex-dependent behavioral effects in the F1 generation, a cross-over breeding design was used to obtain the F1 generation. The treatment crosses included: Control Male x Control Female, Control Male x BaP Female, BaP Male x Control Female, and BaP Male x BaP Female. In the F1 generation following parental 2.5 μg BaP/g fish exposure, there were no significant changes in behavior, similar to their F0 parents. However, in F1 offspring from the 25 μg BaP/g fish parental exposure, there were significant changes (increased total distance traveled and time spent mobile) in the females of the BaP M x BaP F, despite no behavioral effects in their parents. The F1 hyperactivity was only in fish where both parents had been exposed to BaP, and it was only a significant change in females.

Our results indicating persistent hyperactivity coincide with our laboratory findings that behavior of the F1 generation at the larval stage also showed a hyperactive phenotype

(Pandelides et al. 2021). During the larval stage, the sex of the zebrafish cannot be determined. This hyperactivity in the F1 generation is relevant due to the implications it has on BaP exposure during early fetal development in humans. F1 hyperactivity indicates that human exposure to PAHs could result in multigenerational behavioral alterations following parental exposure.

In humans, this is indicative of a child having behavioral effects due to one or both of their parents being exposed before their conception. This is significant due to 52% of the world's population relying on solid fuels as their primary source of energy when cooking, which predominately affects women who in most societies are in charge of cooking (Rehfuess et al., 2006). Not only is cooking a possible source for PAH exposure, but cigarette smoke and industrial processes are as well, indicating that BaP exposure is not uncommon for many women (Services, 1999). Our findings that the F1 females from the BaP M x BaP F cross experience a significant increase in total distance and mobility not only indicate that behavioral impacts of preconceptual BaP are persistent, but they also are sex-dependent. Due to significant changes only being found in the BaP M x BaP F cross, we cannot at this time conclude if males or females in the parental group contribute more strongly to behavioral effects in the offspring. However, in the future, research could be done with increased concentrations of BaP or a more sensitive strain (e.g. AB line) to see if this could be determined. Our findings do indicate that behavioral effects are sex-dependent in the offspring because only females experienced significant behavioral changes. Although further research needs to be done to establish the molecular mechanisms that result in only female offspring being significantly affected, our findings indicate that PAH exposure worldwide may cause multigenerational effects to offspring, specifically in females.

Through Ingenuity Pathway Analysis of the 1153 differentially expressed genes in 96 hpf zebrafish larvae following parental and continuous BaP exposure, it was previously determined that BaP exposure significantly affects the AHR signaling pathway, resulting in differential expression of genes that are regulated by AHR (Fang et al., 2015). In addition to AHR-mediated genes, BaP also affected expression in genes that were not considered AHR-mediated. In order to assess the role of Ahr2 in mediating BaP behavioral toxicity, a similar control and 25 μg BaP/g fish exposure was done in Ahr2-null zebrafish. Previously, Dr. Tanguay's lab analyzed physical and behavioral differences between the Ahr-null and wild-type zebrafish (Garcia et al., 2018). Their study determined that Ahr-null fish have decreased survival and fecundity, and defective dorsal, ventral, and caudal fins. They also discovered visible jaw malformations and notable skeletal abnormalities in females. In order to analyze anxiety-related behaviors, startle response and predator avoidance assays were used. The Ahr-null fish exhibited an atypical predator avoidance response by not appearing to respond to the predator stimulus. To evaluate social behaviors, a social cohesion assay was used to measure the percent of time the fish spent close to a liquid-crystal display (LCD) video projection of a school of free-swimming zebrafish, and the Ahr-null fish were determined to have a significantly increased time near the stimulus. Overall, their study suggests Ahr2 is necessary for some aspects of neuromuscular and/or sensory system development, and the absence of Ahr2 results in impaired behavioral responses in larvae and adult zebrafish (Garcia et al., 2018).

The indication of AHR- associated molecular pathways serving an important role in developmental and cognitive effects led our lab to assess the role of Ahr2 in mediating changes to behavioral responses due to a mechanistic interaction with BaP. In the F0 25 μg BaP/g fish dietary exposure in the Ahr-null zebrafish, there was a significant decrease in total distance

traveled (cm) for the male Ahr2-null zebrafish. This hypoactivity of the Ahr2-null males is a new finding and indicates that there are non-AhR dependent BaP effects on behavior. Importantly, when comparing the 5D and Ahr2-null control groups in open field assays, there were not significant differences in control F0 4 mpf animals. Therefore, the behavioral differences seen were not due to morphological differences in the null animals. Although further research is required, this indicates that BaP is having neurotoxic effects in males that are not AHR-dependent.

Overall, due to the F1 generation of BaP M x BaP F crossed females demonstrating hyperactivity, our study determined that not only are behavioral impacts of preconceptional BaP exposure persistent in F1 adults, but also BaP-induced behavioral impacts are sex-dependent. Also, the hypoactivity of the Ahr-null males indicates that behavioral changes by BaP are not Ahr dependent. Exposure to BaP ultimately resulted in neurotoxicity in the Ahr-null fish but not in the 5D fish. This indicates that Ahr is likely playing a protective role in regards to BaP toxicity by metabolizing the BaP so that it is not bioaccumulated as much; however, it may also be causing DNA damage that could contribute to carcinogenicity over the longer term. Although sex-dependence was important in the F1 exposure, further research is needed to determine the epigenetic mechanisms resulting in female offspring being significantly affected by BaP exposure, as well as to determine if males or females in the parental group being exposed to BaP have a stronger impact on the offspring. Through continued research, our lab will work to compare molecular and physiological effects of BaP in order to develop a better understanding of the impacts of PAH exposure.

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